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# Combinatorial RNAi strategies against HIV-1 and other escape-prone viruses

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**ABSTRACT:**
RNA interference (RNAi) is an evolutionarily conserved mechanism in which double-stranded RNA (dsRNA) induces sequence-specific gene silencing. RNAi as a gene suppression tool holds great promise for basic research and multiple applications. RNAi strategies with a single inhibitor have received much attention and some are currently being tested in clinical trials as potential drug for infectious diseases, cancer and genetic disorders. Although very successful, such an RNAi mono-therapy is not always sufficient. For example, several proteins should be silenced simultaneously for the elucidation of cross-talk between signalling pathways. Furthermore, development of a durable RNAi therapy for viral infections and cancer may also require the use of multiple inhibitors, as this may prevent the selection of virus variants or cancer cells that escape from the RNAi therapy by mutation of the target gene. Therefore, several combinatorial RNAi strategies have been developed and recent reports demonstrated the potency of this approach by targeting multiple viral genes or oncogenes. At the same time, combinatorial RNAi may also increase the risk of saturation of the endogenous RNAi pathway and off-target effects. This review summarizes the latest experimental advances in combinatorial RNAi research and gene therapy approaches, in particular against escape-prone viruses, like the human immunodeficiency virus type 1 (HIV-1).

## INTRODUCTION

### The RNAi mechanism

RNAi was first described as an innate antiviral defense mechanism in plants, insects and nematodes. Viral replication intermediates in the form of dsRNA are recognized and processed by members of the Dicer nuclease family into 21-25 base pair (bp) small interfering RNA duplexes (siRNAs) [1]. This siRNA duplex is incorporated into the RNA-induced-silencing complex (RISC). The "guide" strand programs RISC to cleave the perfectly complementary viral mRNA, whereas the "passenger" strand of the siRNA is degraded.

In mammals, RNAi plays a pivotal role in the regulation of gene expression at the post-transcriptional level through endogenously expressed microRNAs (miRNAs) [2]. These miRNAs are synthesized as primary miRNA (pri-miRNA) transcripts and cleaved by the endonuclease Drosha into RNA hairpins of ~70 nucleotides (nt) known as precursor miRNAs (pre-miRNAs) [3;4]. This pre-miRNA is actively transported from the nucleus to the cytoplasm by Exportin-5, a Ran-GTP cargo transporter, where it is processed by the endonuclease Dicer into miRNA duplexes of ~22 bp [5-7]. One strand of the duplex, the mature miRNA, guides the RISC complex to complementary mRNA sequences, inducing mRNA cleavage in case of perfect base pairing. In case of incomplete base pairing, translational repression may occur. For this, pairing of the first 2-8 nucleotides from the 5' end of the miRNA (seed sequence) to multiple sites in the 3' untranslated region of an mRNA is usually sufficient [8-11]. The role of RNAi as natural antiviral mechanism in mammalian cells is still under investigation [12;13].

The efficiency and sequence-specificity of the RNAi mechanism makes it highly attractive as an approach to knock down disease-associated mRNAs and transcripts encoded by pathogenic viruses [14-16]. In mammalian cells, the natural RNAi pathway can be triggered by transcripts that resemble the pre-miRNA structure: short hairpin RNAs (shRNAs) of 19-21 bp (Figure 1) [17-19]. These shRNAs have been optimized by inclusion of RNA structural motifs that mimic pri-miRNAs (Figure 1) [20-22]. Expression of shRNA constructs is usually driven by RNA polymerase III promoters, including the U6, H1 and several promoters of tRNA genes because of their cellular roles in the production of small endogenous transcripts [17;19;23]. RNA polymerase III

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transcription generally yields high shRNA expression levels and another important advantage over polymerase II transcription systems is the precise initiation and termination site (the latter consists of 4-6 consecutive U residues in the nascent transcript). Instead, an RNA polymerase II promoter is normally used to express miRNA mimics because this type of promoter drives the expression of most natural miRNA genes [24], although polymerase III can also be used to transcribe miRNAs [25]. Advantages of the polymerase II system include the availability of tissue-specific and inducible promoters, which allows regulated expression of the miRNA inhibitor. This may be important to avoid toxicity, in particular because high shRNA expression from the U6 promoter is known to cause fatality in mice due to saturation of the RNAi pathway [26] and activation of the interferon (IFN) system [27]. Furthermore, RNA polymerase II promoters have the ability to transcribe extended transcripts, which makes them very suitable for certain combinatorial RNAi approaches.

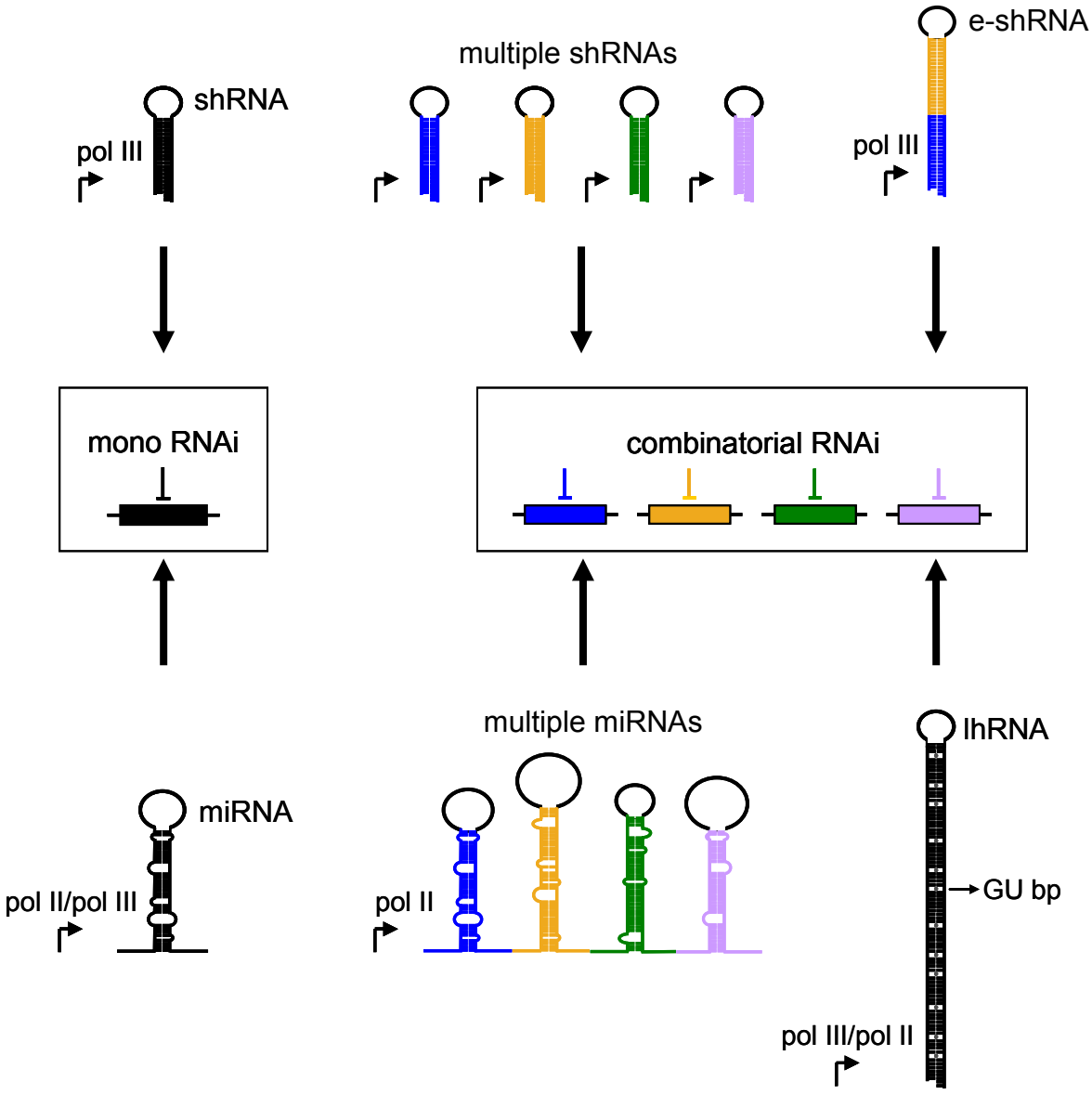


Figure 1: Mono and combinatorial RNAi approaches. Mono-RNAi strategies with an shRNA vector driven by an RNA polymerase III promoter or an artificial miRNA driven by an RNA polymerase II or III promoter. Combinatorial RNAi approaches are shown with multiple shRNA expression cassettes in a single vector, a single e-shRNA, multiple miRNAs expressed from an RNA polymerase II promoter and a lhRNA.

**Why combinatorial RNAi**

Although potent knockdown can be obtained with an optimized single RNAi-inducer, some approaches will require a combinatorial RNAi approach. In specific settings, multiple genes need to be targeted simultaneously or intensified silencing of a single gene is needed to obtain a biological effect. Anti-cancer strategies may need a combinatorial RNAi approach that silences multiple oncogenes at once. Antiviral therapies may require that the efficiency and durability of inhibition is optimized by focusing on multiple targets to prevent viral escape. Furthermore, combinatorial RNAi may be needed in basic research to dissect overlapping functions of redundant protein isoforms, e.g. involved in signalling pathways. The field of combinatorial RNAi against viral targets was reviewed previously by Grimm et al. [28]. This review highlights recent advances in the field of combinatorial RNAi, and we will discuss new methods and novel applications. The sections follow the different approaches: multiple shRNAs, extended shRNAs, long hairpin RNAs and miRNA-like transcripts.

**Combinatorial RNAi by means of multiple shRNAs**

The most straightforward strategy to induce combinatorial RNAi is to simultaneously express multiple identical or different shRNAs from several gene cassettes (Figure 1). This strategy has been used for basic research purposes, for example for dissection of functional roles of partially redundant kinases [29] and transcription factors that induce a signalling pathway [30]. Furthermore, combinatorial RNAi using multiple shRNAs has been used to target different oncogenes in chronic myeloid leukemia (CML) [31-34] and laryngeal squamous carcinoma [35;36]. Another rationale to use multiple RNAi inducers is to intensify the RNAi-mediated suppression of an individual gene. Intensified RNAi was demonstrated using multiple copies of shRNA cassettes targeting a single gene [37-40].

Other interesting targets for combinatorial RNAi therapy include escape-prone viruses such as poliovirus, HIV-1, hepatitis B virus (HBV) and hepatitis C virus (HCV). RNAi mono-therapy against these viruses faces the same obstacle as mono-therapy with antiviral drugs, that is the selection of viral escape variants [41-45]. The sequence-specificity of RNAi now turns into a disadvantage because a single point mutation in the target is sufficient to abolish RNAi-mediated inhibition. Viral escape was described by Gitlin et al. [41;42] in poliovirus-infected cells treated with siRNAs. Similarly, HCV escape mutants with multiple point mutations in the target sequence emerged upon successive mono-siRNA treatment in the HCV replicon system [43]. Several groups reported the emergence of HIV-1 variants with a point mutation in the target sequence upon elicitation of RNAi pressure by a single inhibitor [44-46]. Interestingly, HIV-1 can also escape by introducing a single nucleotide mutation upstream of the target sequence that triggers a change in local RNA secondary structure of the target [47], although this may represent a rather unique escape route [48]. This induced RNA structure occludes the target sequence for binding to the complementary siRNA, thus explaining the resistance phenotype [49].

Thus, long-term inhibition of viruses that establish a chronic infection in the host is not likely obtained using a mono-RNAi therapy. To obtain a permanent block of virus replication, a combinatorial attack is required that simultaneously targets several viral sequences. Ideally, such targets should encode essential functions and its sequence should be well conserved among different virus strains, thus avoiding that genetic variation will impair the therapy [50]. Another therapeutic possibility against escape-prone viruses is to knock down cellular cofactors that are essential for viral replication, e.g. mediating the process of viral entry by targeting the receptor molecules. Peripheral blood mononuclear cells (PBMCs) that stably co-express shRNAs against the HIV-1 co-receptors CXCR4 and CCR5 were protected against viral infection [51]. The therapeutic potential of such inhibitors has to be carefully validated to exclude the possibility that novel HIV-1 variants are selected that use new receptors for cell entry.

Chang et al. [52] used lentiviral vectors encoding anti-HIV shRNAs against highly conserved sequences in the pol, int and vpu genes and demonstrated efficient inhibition of virus replication in acutely and chronically infected cells and PBMCs. The combination of 3 vectors was much more efficient than treatment with an individual shRNA vector. However, the possible

prevention of viral escape was not evaluated in this study.

We performed a study using 86 shRNA gene constructs against highly conserved HIV-1 sequences. Multiple effective shRNAs against 8 targets within the HIV-1 RNA genome were identified. Furthermore, these shRNAs were validated by confirming sequence-specific inhibition in independent luciferase reporter assays. Virus production in cells that stably express 3 shRNAs from a single lentiviral vector was strongly reduced compared to the cells expressing a single shRNA. Furthermore, cells that stably express 2 shRNAs showed a more durable inhibition compared to cells expressing a single shRNA construct. In a follow-up study [53], different promoters were used to express 4 shRNAs against HIV-1. This approach was used to prevent recombination-mediated deletion of shRNA cassettes on repeated H1 promoter sequences [54]. In contrast to cells expressing a single shRNA, no HIV-1 escape was observed in 4-shRNA transduced cells. Notably, these results were obtained with a considerable dose of challenge virus in stably transduced cells with only a single copy of the therapeutic vector. A low shRNA expression level is important to avoid the risk of toxicity due to saturation of the RNAi pathway. These results indicate that a combinatorial RNAi approach against HIV-1 could be safe and effective, but further studies are needed to validate the therapeutic potential *in vivo*.

To induce combinatorial RNAi against HCV, Henry et al. [55] constructed 2 and 3-shRNA lentiviral vectors targeting two regions of the HCV genome and the host cell receptor, CD81. These vectors specifically inhibited HCV replication for approximately 17 days. Interestingly, a 2-shRNA vector targeting a host factor (CD81) and an HCV gene (NS5B) showed reduced efficacy compared to vectors that target 2 viral sequences (NS5B and IRES). Whether this reduced efficacy is perhaps caused by a relatively poor efficacy of the individual shRNAs remains to be tested.

Interestingly, some mammalian viruses exhibit a close interaction with the cellular RNAi pathway, e.g. they use cellular miRNAs for their own benefit or they encode their own miRNAs [56]. Furthermore, several viruses encode RNAi suppressors to inhibit the antiviral RNAi response [14]. Whether the presence of these RNAi suppressors will affect the antiviral RNAi effect is currently unknown. The initial results indicate that this is not a serious problem for RNAi therapeutics. For instance, we and others described potent HIV-1 inhibition despite the presence of the Tat protein that has been shown to exhibit RNAi suppressor activity [57;58].

Taken together, these results demonstrate the effectiveness of combinatorial RNAi by multiple shRNA expression from the same or different promoters. The use of identical promoters in a single vector should be avoided as it can cause recombination or deletion due to sequence similarity. To circumvent this problem, different promoters can be used for the expression of multiple hairpin RNAs. However, a careful selection of promoters is necessary to ensure an approximately equal expression level of each hairpin. Unequal RNAi pressure on different target sites may not be ideal to prevent viral escape. Important safety issues including targeting of other genes (off-target effect) and saturation of the RNAi pathway need to be analyzed in detail to determine the clinical usefulness of this approach.

**Combinatorial RNAi by means of extended shRNAs**

To induce combinatorial RNAi against HIV-1, we combined 2 highly effective shRNAs targeting 2 conserved regions in a single extended shRNA (Figure 1, e-shRNA) transcript expressed from an H1 promoter [59]. This approach of shRNA-stacking requires a very careful design, because the guide strands of the 2 shRNAs do not target a contiguous sequence of the target mRNA. Thus, imprecise Dicer cleavage will generate a second siRNA that is not perfectly complementary to the target, and thus loosing effectivity. We made a set of e-shRNAs with increasing stem length and observed that the first siRNA that is made from the base of the hairpin stem is always generated and fully active. However, the second siRNA was only produced when the hairpin stem reached a certain length. Ongoing studies indicate that such stacked e-shRNA constructs are active with a stem length up to approximately 66 bp, which means it can maximally encode 3 different siRNAs (Liu et al. unpublished results). Processing of the optimized e-shRNA molecules seemed equally effective as the respective individual shRNAs. Importantly, e-shRNAs do not induce an IFN

response in 293T cells at the efficacious dose. Although e-shRNA expression vectors may present promising approaches for durable inhibition of escape-prone viruses, their potential to prevent viral escape should be studied in more detail. In particular, the safety of these vectors should be determined further in appropriate *in vivo* models.

**Combinatorial RNAi by means of long hairpin RNAs**

Another method to induce combinatorial RNAi is to express long hairpin RNAs (Figure 1, lhRNA) driven by RNA polymerase III or II promoters. These transcripts will be processed into multiple siRNAs. In contrast to transfection of dsRNA molecules larger than 30 bp, intracellular expression of an effective dose of lhRNA does not readily elicit an IFN response [60]. Furthermore, inclusion of point-mutations in the sense strand of the hairpin that result in G-U base pairs can avoid this immunostimulatory effect [61;62]. Thus far, lhRNA expression vectors have been used to inhibit several escape-prone viruses including HIV-1, HBV and HCV [46;60-64]. Efficient knockdown of HIV-1 was reported using lhRNAs of 50, 53, 60, 80 and 300 bp without inducing an IFN response [46;60;63;65]. We measured reduced anti-HIV activity for a 300-bp lhRNA compared to a regular shRNA-inhibitor [60], although most of the inhibitory capacity of this particular lhRNA transcript may be due to a non-RNAi mechanism that is induced by sense HIV-1 leader sequences [66]. Efficient inhibition of HCV was also demonstrated using 50, 100 and 197-bp hairpins with mutations in the sense strand [61;62]. Importantly, these lhRNAs can silence both wild-type and shRNA escape viruses, indicating that multiple effective siRNAs were generated from the single lhRNA precursor [46;60;62].

Akashi et al. [61] were the first to demonstrate that a 50-bp hairpin indeed produces 2 siRNAs. A detailed analysis of a 62-bp lhRNA against HBV by Weinberg et al. [64] revealed that siRNAs are mainly produced from the base of the hairpin. Consistent with these results, anti-HIV lhRNAs of 50, 53, 69 and 80 bp also generated a gradient of siRNAs from the base to the loop of the hairpin through Dicer-mediated cleavage [65;67]. Interestingly, cells that stably express the anti-HIV lhRNAs were shown to be more effective suppressors of HIV-1 than a single shRNA in long-term culture experiments [65], although no viral escape studies have yet been reported.

Compared to a simple shRNA inhibitor, the lhRNA encodes additional siRNAs that target mRNA sequences that are immediately adjacent to the sequence targeted by the initial siRNA derived from the base of the lhRNA. This lhRNA design has the main advantage that any siRNA produced from the lhRNA, independent of the precise position of Dicer cleavage, will be fully complementary to the target mRNA. An obvious disadvantage is that not all processed siRNAs will turn out to be effective inhibitors. This disadvantage is solved in the e-shRNA design, which combines highly active siRNAs in a single transcript by stacking.

**Combinatorial RNAi by means of miRNAs**

Another, perhaps more elegant strategy to induce combinatorial RNAi is to express a single transcript that encodes multiple artificial miRNAs (Figure 1). These transcripts resemble a natural miRNA cluster that is transcribed as a single transcription unit (polycistron) from an RNA polymerase II promoter. This strategy has been used in several studies for different purposes: to inhibit multiple viral genes, to intensify RNAi and to knock down disease-associated genes.

Since miRNAs require a two-step processing by Drosha in the nucleus and by Dicer in the cytoplasm, several groups studied the RNAi effect by chaining several miRNA elements. A modified miR-30 transcript was designed by Zhou et al. [68] to target the mRNA for human superoxide dismutase 1 (SOD1), which is mutated in the neurological disorder amyotrophic lateral sclerosis [69]. To intensify the RNAi effect, 2 miRNA-like hairpins were concatenated in a single transcript (separated by 100 nt) and expressed from the cytomegalovirus (CMV) immediate early promoter. Surprisingly, inhibition by the 2-miRNA vector was decreased compared to the single miRNA vector. This could be due to processing problems of the tandem-miRNA transcript, which highlights the importance of a careful design when multiplexing different miRNAs.

In contrast to the previous findings, Sun et al. [70] reported increased siRNA levels and a more profound inhibition when 2 miR-30 based hairpins were concatenated compared with a single

miRNA. However, addition of a third hairpin only modestly increased the RNAi activity. Interestingly, the inhibitory activity of a single hairpin is even enhanced upon multimerization with an irrelevant second hairpin, arguing for a general effect on transcript stability or increased efficiency of miRNA processing. Consistently, Chung et al. [71] observed a gradual increase in RNAi activity using up to 8 miR-155 modified miRNAs against the luciferase mRNA reporter.

A recent report by McLaughlin et al. [72] showed the efficacy of combinations of miRNA mimics targeting several sites within the Abl-coding sequences for the treatment of leukemia.

Previously, this group studied the potential of anti-Bcr-Abl shRNAs to suppress leukemic cell growth *in vivo*, but significant levels of Bcr-Abl kinase activity remained. Using a lentiviral vector encoding 3 anti-Abl miRNAs, oncogene expression and activation of the Bcr-Abl pathway could be effectively suppressed. Importantly, the suppression is in such an extend that regrowth of leukemic cells is prevented *in vitro* and *in vivo*. Besides the ability to co-express multiple miRNAs, another major advantage of using an RNA polymerase II promoter is the possibility for conditional RNAi [73]. For example, Shin et al. [74] expressed artificial miRNAs from a lentiviral vector using a tetracycline-regulated promoter. Conditional knockdown of the heterotrimeric G proteins Gα12 and Gα13 was obtained using this vector type encoding 2 artificial miRNAs.

Recently, Snyder et al. [75] expressed anti-HBV miRNAs under control of a liver-specific promoter. A polycistronic construct encoding 4 different anti-HBV miRNAs was generated and demonstrated to be efficient in inhibiting HBV production. Sequence-specific inhibition by each antiviral miRNA was tested on independent luciferase reporter constructs. Modest inhibition was obtained per miRNA, suggesting that the potent inhibition of HBV production was an additive effect of all antiviral miRNAs.

We used a multiplex miRNA expression strategy based on the mir-17-92 cluster to target HIV-1 at different highly conserved regions [22]. First, we generated a large set of anti-HIV-miRNAs and selected the best inhibitors for construction of the polycistronic construct. Second, we generated constructs encoding 2, 3 and 4 hairpins. Interestingly, consistent with the results from Sun et al. [70], we observed an enhanced inhibitory activity of a miRNA when it is concatenated to other, even irrelevant hairpins in a single transcript. The increased inhibitory effect correlated well with increased production of the mature miRNAs. Our results indicated that the intrinsic activity of a miRNA is in fact higher than that of an shRNA. Furthermore, we showed that HIV-1 replication is inhibited in T cell lines that stably express 4 antiviral miRNAs.

In summary, these results indicate that the multi-miRNA approach can be used in combinatorial RNAi therapeutics. However, careful design for concatemerization of the hairpins is necessary to ensure proper processing and optimal inhibitory activity. Further studies are required to test the impact of many variables (hairpin number, hairpin positioning within polycistron, spacer length between hairpins, type of miRNA scaffold etc.) to optimize this approach.

**Side effects and off-target effects of combinatorial RNAi**

To apply combinatorial RNAi in a therapeutic setting, critical issues including safety and efficacy have to be carefully addressed. Safety is the major concern, since even a mono-RNAi treatment can produce an unwanted side-effect, including IFN induction [76], off-target effects on other mRNAs [77] and toxicity due to saturation of the endogenous RNAi pathway [26]. Such risks are likely to increase in a combinatorial RNAi setting, in particular the off-target effect and saturation of the RNAi pathway. Off-targeting can be due to pairing of the seed area of the siRNA/miRNA guide strand with complementary sequences in the 3' UTR of non-target mRNAs. To minimize the risk of toxicity, the concentration of RNAi inducer should ideally be fine-tuned. The use of a cell-type specific polymerase II promoter has several advantages in this respect. First, expression of the RNAi inducer will only occur in the cells of interest. Second, it will allow regulated shRNA/miRNA expression. Recently, miRNA-like hairpins were shown to be more active than simple shRNAs, thus allowing the use of a lower dose [21;22]. Interestingly, the miRNA-scaffold also resulted in significantly reduced neurotoxicity in mice compared to the respective shRNA [78]. Thus, artificial miRNAs may provide a suitable approach to enhance RNAi activity and simultaneously reduce toxicity. However, further

studies are needed to elaborate on these initial findings. To minimize the toxicity and off-target effects, it is also important to identify the best mode of gene delivery [79]. The concerns regarding combinatorial RNAi need to be carefully addressed using the lowest efficacious dose in appropriate *in vivo* animal models.

## Conclusion and perspective

Combinatorial RNAi has great potential to attack escape-prone human pathogenic viruses such as HCV and HIV. Preliminary studies also show the potency for the treatment of metabolic, genetic and blood disorders. Furthermore, combinatorial RNAi may provide an effective tool to unravel roles of different redundant factors in complex cell signalling and transformation processes. Although combinatorial RNAi is very attractive, further *in vivo* studies must be carried out to analyze possible side effects.

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